Epoxidation of Aldrin by Excised Pieces of Plant Tissue

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Aldrin applied to tissue pieces of field-grown radishes, beets, turnips, rutabaga, carrots, and parsnips was converted to dieldrin. The epoxidation rates were different for different crops, but varied little for the same plant species grown in two consecutive years and treated and incubated in the laboratory under identical conditions. The reaction rates remained constant with time between 24 and 72 hours of incubation. Destruction of the cell structure by grinding or freezing prevented the formation of dieldrin. Heat, very low partial pressure of O_2 during incubation, and addition of KCN and 2,4-DNP also inhibited the reaction. Aldrin applied to the surface of roots or stems of young pea plants (5 to 7 days of germination) resulted in the production of dieldrin. Reaction rates were significantly higher with roots than with stems; most activity was noticed with root tips and least with the upper parts of pea root.

Research on the metabolism of the cyclodiene insecticides by plants has primarily been carried out with entire plants (Edwards, 1966; Finlayson and MacCarthy, 1965; Lichtenstein *et al.*, 1967; Marth, 1965; Spencer, 1965). The use of a more simplified plant system, such as isolated pieces of plant tissues, would make it possible to establish more definitely if plants catalyze reactions of cyclodiene insecticides and to investigate more closely reaction rates and mechanisms, and the factors influencing them.

Early work with chlorinated hydrocarbons and their fate after application to plants was primarily concerned with persistence, penetration, and translocation of the parent compounds (Gunther and Blinn, 1955; Nolan and Wilcoxon, 1950). Chemical changes, including the conversion to other toxic substances, were reported later (Beck *et al.*, 1962; Edwards *et al.*, 1957; Gannon and Decker, 1958; Glasser *et al.*, 1958; Lichtenstein, 1959, 1960; Lichtenstein and Medler, 1958; Lichtenstein and Schulz, 1960, 1965; Lichtenstein *et al.*, 1964, 1965a, b, 1967; Terriere and Ingalsbe, 1953). All these results denote a system in plants which catalyzes aldrin epoxidation. This may be one of the mixed-function oxidases which, according to Metcalf (1966), abound in plants and are responsible for aldrin epoxidation.

Oxidative enzymes found in the microsomes of liver (higher animals) (Gillette, 1963) and insects (Nakatsugawa *et al.*, 1965) epoxidize aldrin. Evidence that intact plants can also catalyze this reaction is very strong according to the above investigations. The following study was conducted to provide under controlled laboratory conditions further evidence that isolated plant systems catalyze aldrin epoxidation.

MATERIALS AND METHODS

Plant Materials. Field Crops. Radishes (Scarlet Globe), red beets (Detroit Dark Red), turnips (Purple Top Strap Leaf), rutabaga (Laurentian), carrots (Red Cored Chantenay), and parsnips (All American) were grown during 1965 and 1966 in the field in an insecticide-free Carrington silt loam soil. They were usually seeded in late May and grown, in 30-foot rows, to a stage which would have qualified them for marketing. In 1965, radishes were also grown during July and August. Some samples of 1965 crops were also grown beyond marketing age as part of experimental conditions.

Laboratory-Grown Peas. Peas (Alaska Wilt Resistant) were grown in the laboratory. To obtain high germination rates, seeds were surface-sterilized by submerging them in 0.5% aqueous sodium hypochlorite for 10 minutes (water-diluted Chlorox, Chlorox Co., Oakland, Calif.), followed by thorough rinsing with water (Sanwal, 1963). Washed and sterilized conical clay pots (opening 15-cm. I.D.) were filled with water-saturated vermiculite, and about 100 seeds per pot were placed in the vermiculite. The pots were placed in clay saucers to which water was added as required. The peas were grown under GRO-LUX lamps ($T = 22° \pm 2°$ C., RH = 40 ± 5%, 9 hours of light per day) until the roots were 5 to 8 cm. long, usually for 5 to 6 days, and then processed as described below.

Chemicals. Redistilled acetone and hexane were used for extractions and standard solutions of the insecticides. Analytical grades of aldrin and dieldrin were used for treatment (dissolved in acetone) and as references during analytical procedures (dissolved in hexane). The 0.02*M* phosphate buffer, pH 6.5, was prepared with deionized water according to Gomori (1955).

Application of Insecticides and Incubation. Tissue Cubes of Field Crops. The edible portions of the six root crops were cleaned according to Lichtenstein (1959). To reduce microorganismic contamination of the tissue pieces, all equipment was heat-sterilized and the stainless steel knife used for cutting was kept in 70% aqueous ethanol. The roots were cut into cubes of approximately 8 cu. mm. (2 × 2 × 2 mm.); 8-gram portions were weighed into round glass jars (90-ml. volume, 3.4-cm. I.D.), which were then covered with aluminum foil.

One hundred micrograms of aldrin were applied to each 8-gram portion of tissue cubes by distributing 0.2 ml. of a solution of aldrin in acetone as evenly as possible by means of a glass syringe (0.25-ml. capacity). The aluminum foil was replaced as quickly as possible in order to minimize air-borne microbial contamination. The cubes were then mixed by shaking, followed by incubation at 35° C. in a

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water vapor–saturated atmosphere for 24, 48, or 72 hours. At the end of the incubation periods, the reaction was stopped either by immediate extraction or by freezing at −23° C. until extraction.

PARTS OF LABORATORY-GROWN PEAS. Peas, grown in the laboratory, were removed from the clay pots and the roots were gently tapped to remove adhering particles of vermiculite. By use of a graduated syringe (10 μl.), aldrin in acetone (1000 μg. per ml.) was then applied topically to roots or stems at 5 μl. per root or stem, resulting in an application of 5 μg. of aldrin per root or stem. Plants were left intact during incubation. In experiments with excised roots, the aerial parts of the plants were discarded prior to treatment and incubation.

Since it is desirable to achieve even distribution of aldrin over the entire root surface in order to obtain reproducible and comparable results, the method of application was modified in some experiments. In these, the excised roots were briefly (0.5 to 1 second) submerged in an acetone solution containing 200 μg. of aldrin per ml., then exposed to a stream of air in order to evaporate the acetone without undue delay. The amount of aldrin applied to each root by this application method remained unknown, and could have varied from root to root.

Incubation was carried out at room temperature. Ten treated seedlings, or roots, were placed in a Petri dish in the presence of water-saturated filter paper in order to prevent desiccation. The Petri dishes, each containing one sample, were stored in light or darkness for various periods of time. Reactions were terminated by extracting the roots for analyses.

Analytical Procedures. EXTRACTIONS. Tissue Cubes of Field Crops. Fifty milliliters of a 1-to-1 mixture of hexane and acetone were added to the 8 grams of tissue cubes inside the incubation jars. The plant material was then macerated within the same jars for 15 minutes with a Lourdes homogenizer (Volu-Mix, Lourdes Instrument Corp.) at medium speed. After that, the acetone was removed by washing with one 50-ml. portion of water and two 50-ml. portions of a 2% aqueous solution of sodium sulfate. Aliquots of the hexane phase were then pipetted into tightly stoppered glass vials containing about 0.5 gram of anhydrous sodium sulfate and stored at −23° C. for future analyses.

Parts of Laboratory-Grown Peas. Ten roots of one sample were cut into short pieces and ground in a porcelain mortar in the presence of a few milliliters of a 1-to-1 mixture of hexane and acetone. Fifty milliliters of this mixture were quantitatively transferred into a glass-stoppered Erlenmeyer flask.

The Erlenmeyer flasks were shaken on a reciprocating shaker at 140 excursions per minute. After 1 hour of shaking, the contents were filtered into separatory funnels for acetone removal by water washes as described above. The recovered hexane phase was not brought up to volume, so that results may have been too high, if hexane had evaporated during extraction.

ANALYSES. All extracts were analyzed for aldrin and dieldrin by gas-liquid chromatography (GLC). Three instruments, each equipped with an electron-capture detector, were used.

A Jarrell-Ash gas chromatograph, Model 28-700, equipped with a 100-mc. electron affinity ionization detector, operated at 20 volts. The 1.22-meter long glass column (4-mm. I.D.) contained 60- to 80-mesh Chromosorb W, A/W, DMCS, as solid phase, coated with 5% DC-11. Nitrogen was used as the gas phase at 20 p.s.i. (flow rate 125 ml. per minute) and 190° C. Injector temperature was 250° C., that of the detector 210° C.

A Jarrell-Ash gas chromatograph, Model 700, with components and operating conditions described above.

An Aerograph gas chromatograph, Model 700, equipped with a 250-mc. tritium electron affinity ionization detector, operated at 90 volts. Column and other operating conditions were similar to those described above, except that the nitrogen flow was 50 ml. per minute.

Standard solutions containing aldrin and dieldrin in hexane were used to prepare standard curves each day an analysis was performed. Each experiment contained aldrin- and dieldrin-free control samples, in order to establish that no interfering substances were present.

RESULTS AND DISCUSSION

Tissue Cubes of Field Crops. EPOXIDATION RATES OF DIFFERENT CROPS AND FACTORS INFLUENCING THEM. Table I shows that isolated pieces of plant tissue contain a system which oxidizes the insecticide aldrin to its toxic analog dieldrin and that the epoxidizing activity varies with the plant species: Of the six crops tested, red beet tissue was most active under the experimental conditions, while radish tissue had the lowest conversion rates. Furthermore, activity does not vary much for tissue of the same plant species if grown under comparable conditions in the field, and if treated and incubated under identical conditions in two consecutive years.

Potatoes contain especially high proportions of dieldrin if grown in aldrin-treated soil, as demonstrated over several years by Lichtenstein and Schulz (1965). Attempts were unsuccessful to test the tissue of potatoes in the laboratory under the same experimental conditions as the above six root crops. Tissue cubes of potatoes, prepared in the same manner as the other root crops, always darkened quickly and mostly disintegrated during incubation. Consequently, no reproducible or meaningful results could be obtained with potato tissue cubes prepared and treated in the laboratory.

Table I. Epoxidation of Aldrin

Expressed as dieldrin produced in % of aldrin plus dieldrin recovered, by excised pieces of root tissue obtained from field-grown root crops seeded in late May and harvested at marketing age. Applied 100 μg. of aldrin per 8-gram sample of tissue pieces. Incubated 48 hours at 35° C.

	Grown during		1965 + 1966
	1965	1966	2
Red beets	54.7 ± 5.7(3)[a]	46.0 ± 7.2(6)	50.4 ± 4.4
Carrots	43.7 ± 1.1(3)	43.6 ± 2.4(6)	43.7 ± 0.1
Turnips	39.0 + 8.2(3)	37.2 ± 2.6(6)	38.1 ± 0.9
Parsnips	...	37.8 ± 2.4(6)	37.8
Rutabaga	35.4 ± 0.7(3)	38.7 ± 2.2(6)	37.1 ± 1.7
Radishes	19.7 ± 3.5(4)	17.1 ± 0.8(6)	18.4 ± 1.3
	24.6 ± 0.6(5)[b]		

[a] SD, followed by number of replicates in brackets.
[b] Seeded in July and grown for 31 days.

INCUBATION TIME. The effect of incubation time was investigated with tissues from red beets, carrots, rutabaga, and radishes. Eight grams of tissue cubes were treated with 100 μg. of aldrin as described and incubated for 24, 48, and 72 hours. Analytical results (Figure 1) indicated that the four crops epoxidized aldrin at various rates and that the increase of dieldrin production with time was linear within the studied time interval.

The time interval from 0 to 24 hours was not examined. Extending the straight lines to 0 hour—i.e., to the beginning of the incubation period—would not lead through the point of origin. The lines would intersect the ordinate above zero. It can be concluded that the rate of product formation must have been higher between 0 and 24 hours of incubation before leveling off and becoming linear.

This may be due to a change in distribution of aldrin within the cubes and resulting change in oxygen availability: Initially most of the substrate was near the surface of the cubes and possibly sufficient oxygen and enzyme were present to yield higher dieldrin formation, while oxygen availability may have become the limiting factor with progressing aldrin transport across cells into the inner parts of the cubes.

This explanation assumes that aldrin epoxidation is catalyzed by mixed function oxidases utilizing atmospheric O_2 as described by Ingraham (1962) and Graf and Fallab (1964), and that availability of O_2 is the limiting factor after aldrin has penetrated into the inner parts of the cubes.

If such factors as product inhibition or substrate depletion had been responsible for the rate decrease between 0 and 24 hours, the rate should not have been constant between 24 and 72 hours.

CELL DESTRUCTION. Both freezing and grinding of tissue interfere with the integrity of cell structure. To study influence of these two factors on the activity of tissue, the tissues of red beets, carrots, turnips, and radishes were frozen (−23° C.) prior to aldrin treatment and incubation. In no case could dieldrin be demonstrated.

In addition, tissue of red beets was ground with a household meat grinder, but the resulting pulp was inactive, since no dieldrin could be detected. Radishes were peeled, followed by separate grinding of the cores and peels. Again, no dieldrin could be detected after incubation with aldrin for 48 hours at 35° C. Homogenizing whole radishes in the presence of water or buffer (0.02*M* phosphate, pH 6.5) with a Lourdes homogenizer (Volu-Mix, Lourdes Instrument Corp.) also destroyed all activity. Aerating the aldrin-treated homogenate during incubation did not yield dieldrin.

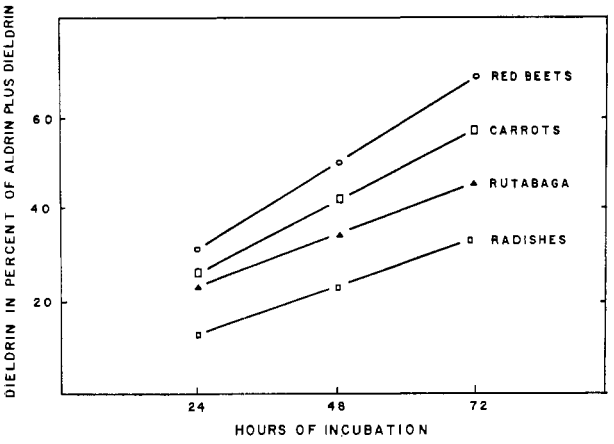

Figure 1. Effect of incubation time on dieldrin formation by 8-gram portions of excised tissue pieces obtained from field-grown root crops

Applied, 100 μg. of aldrin per sample

Plant cells, unlike animal cells, are vacuolated. Large proportions of acids which occur plentifully in plants are contained in these vacuoles (Ranson, 1965). It is possible that interfering with the cell structure of these tissues releases vacuolated substances which inhibit the epoxidation.

HEAT. If the tissues of the root crops were boiled for several minutes in water prior to aldrin treatment and incubation, no dieldrin was formed. Probably heat denaturation of catalytically active proteins had occurred.

ATMOSPHERIC OXYGEN. The role of atmospheric oxygen was tested in two ways. The 8 grams of tissue cubes were either incubated in an atmosphere with very low partial pressure of oxygen or submerged in water. In the first case, the cubes were treated with 100 μg. of aldrin, then the glass jars were purged as much as possible with nitrogen for several minutes, after which they were sealed with four layers of polyethylene film. Incubation was carried out at 35° C. for 48 hours as usual.

The results (Table II) show that no dieldrin could be demonstrated in samples in which the tissue cubes were incubated while submerged under water or a buffer solution. A nitrogen atmosphere inhibited the epoxidation to various extents; in red beets the activity was decreased by about 70% in the presence of nitrogen, in radishes by 94%, and no dieldrin could be detected in turnip tissue.

That complete inhibition could not be obtained in all samples incubated under nitrogen was probably due to a leak in the polyethylene cover or to incomplete displacement of oxygen. Not much of the oxygen dissolved within the tissue would have been removed during the short period during which the glass jars were flushed with nitrogen.

No epoxidation could be demonstrated in the samples submerged under water or buffer. This is somewhat surprising if one considers the absolute amounts of aldrin and oxygen involved. To epoxidize 50% of the applied

Table II. Effect of Atmospheric Oxygen on Epoxidation of Aldrin by Excised Pieces of Root Tissue Obtained from Field-Grown Root Crops

(Applied, 100 μg. of aldrin per 8-gram sample of cubes)

	Incubated in	Recovered, D, % of A + D
Red beets	Water	0
	N_2-atmosphere[a]	10.3
	Air (control)	32.0
Radishes	Buffer	0
	N_2-atmosphere[a]	1.5
	Air (control)	25.0
Turnips	N_2-atmosphere[a]	0
	Air (control)	28.2

[a] Jars were purged with nitrogen for several minutes prior to incubation.

A = aldrin, D = dieldrin.

aldrin (50 μg.), 2.2 μg. of oxygen are required. If copper-containing mixed function oxidases catalyze the reaction, as suggested by Metcalf (1966), only one oxygen atom of atmospheric O_2 would be transferred to the substrate, so that 4.4 μg. of atmospheric oxygen would be necessary to epoxidize 50 μg. of aldrin. Water dissolves approximately 0.29 ml. of O_2 per 100 ml. of water at 37° C. and a partial pressure of O_2 of 95 mm. of Hg (Guyton, 1961). This is equivalent to about 4 μg. of oxygen per 1 ml. of water at these conditions. Considering that the water containing the cubes was in equilibrium with the atmosphere (about 150 mm. of Hg of oxygen) and that the incubation time was 48 hours, sufficient oxygen should have been available. It is possible, however, that tissue respiration competes so successfully for the dissolved and diffusing quantities of oxygen that epoxidation is inhibited by submerging the tissue in water.

ENZYME INHIBITORS. The effects of two inhibitors of oxidative phosphorylation were also tested. Tissue cubes were submerged for 15 minutes in aqueous solutions of (0.05*M* and 0.002*M*) potassium cyanide or 0.02*M* and 0.002*M* 2,4-dinitrophenol. After 15 minutes they were taken out and excess liquid was removed by placing the cubes on facial tissue prior to putting 8-gram aliquots into glass jars. Aldrin treatment and incubation followed as usual. Both KCN and DNP inhibited aldrin epoxidation completely under the experimental conditions if used at 0.05*M* and 0.02*M*, respectively. DNP inhibited the reaction completely at 0.002*M*, but KCN did not inhibit epoxidation entirely at 0.002*M* (Table III). Since transport across several cell membranes may be involved under these experimental conditions, the hydrophilic KCN may not have reached all metabolically active sites of the tissue cubes while the lipophilic substrate aldrin did.

Parts of Laboratory-Grown Peas. LIGHT. The influence of light during incubation was tested by exposing plants to light during incubation with aldrin. Plants were obtained as described and treated with 5 μg. of aldrin per stem, 10 plants per sample, and incubated in light (GRO-LUX) or dark within glass-covered Petri dishes. Results are, expressed as dieldrin in per cent of the recovered total, 19.5 ± 2.1 for dark incubation and 22.8 ± 2.8 for light incubation. A *t* test showed that these results are not significantly different at the 95% probability level.

PART OF PEA PLANT. Aldrin was topically applied with a microsyringe at 5 μg. per root and 5 μg. per stem. Ten plants were used per sample. The samples were duplicated and incubated for 48 hours as described. The results showed that the activity was higher for roots (31.5 ± 3.3 dieldrin in per cent of recovered aldrin plus dieldrin) than for stems (18.4 ± 1.7), as evidenced by a *t* test for the 95% probability level.

PART OF PEA ROOT. To determine the distribution of the aldrin-epoxidizing factor in pea roots, 20 roots each were treated topically with a syringe, and another 20 were dip-treated as described. They were then incubated for 1 hour, after which each root was divided into the root tip, the root-hair zone, and the remaining upper part of the root. The three root zones were then extracted separately. Root tips were most active in converting aldrin to dieldrin (Table IV).

The root tips consist mainly of meristem and the zone of elongation (Wilson and Loomis, 1962). Metabolic activities, as indicated by respiratory rates, are highest in this area of the root. Also, the tip area contains the highest proportion of protoplasm (Meyer, *et al.*, 1960). The root-hair zone, roughly equivalent to the area of maturation, contains mainly differentiated tissue and a lower proportion of protoplasm. Thus epoxidation rates for aldrin applied to the different zones of roots of young

Table III. Effect of Enzyme Inhibitors KCN and 2,4-DNP on Epoxidation of Aldrin by Excised Pieces of Root Tissue Obtained from Field-Grown Root Crops

	Inhibitor	D, % of A + D
Turnip	0.05*M* KCN	0
	0.02*M* DNP	0
	None	28.2
Radish	0.002*M* KCN	6.1
	0.002*M* DNP	0
	0.002*M* KCl[a]	20.5
	None	21.9

[a] KCl used as control.
A = aldrin, D = dieldrin.

Table IV. Distribution of Aldrin-Epoxidizing Factor along the Roots of Young Pea Plants

(Rates of epoxidation by different root zones)

Treatment	Root Zone	D, % of A + D
Topical	Tip	26.3 ± 0.1 = 100%[a]
	Hair	17.6 ± 1.9 = 67%
	Upper[b]	3.4 ± 0.8 = 13%
Dipping	Tip	13.9 ± 0.9 = 100%
	Hair	9.6 ± 1.2 = 70%
	Upper[b]	3.5 ± 0.1 = 25%

[a] % of root tip activity.
[b] Remainder of root after removal of tip and hair zone.
A = aldrin, D = dieldrin.

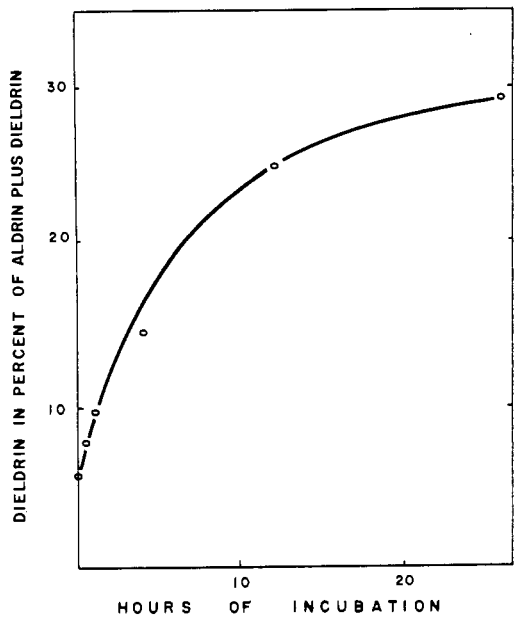

Figure 2. Effect of incubation time on dieldrin formation by excised roots of laboratory-grown peas

pea plants corresponded to the general metabolic activities of the different root zones.

INCUBATION TIME. The effect of incubation time was studied with excised roots to which aldrin was applied by dipping as described. Three replicates consisting of 10 roots per sample were incubated at 35° C. for 0.5, 1, 4, 12, and 26 hours (Figure 2). Epoxidation begins rapidly but does not increase linearly for more than about 1 hour, after which time rates of aldrin epoxidation begin to decrease. Considering the small amounts of aldrin applied by the dipping method—the total amounts of aldrin plus dieldrin recovered per 10-root sample varied from about 15 to 25 μg.—substrate depletion could easily account for the result. Comparison with the time study with tissue cubes of field-grown crops is very difficult because of the different experimental conditions.

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